

**A HUMAN *LACTOFERRIN* PRODUCED BY USING AN INSECT CELL AND
METHOD USING THE SAME**

TECHNICAL FIELD

5 The present invention relates to human *lactoferrin* produced by using an insect cell and a method producing the same. More particularly, the present invention relates to human *lactoferrin* produced by using an insect cell and a method producing the same, in which the method includes the steps of transducing a human *lactoferrin* gene into an insect cell by gene recombination, cloning and expressing human *lactoferrin* in the insect
10 cell, and producing the human *lactoferrin* by using the insect cell.

Human *lactoferrin* is a member of the transferrin family of iron-binding monomeric glycoproteins and also called "*lactotransferrin*". Such a human *lactoferrin* exists in milk of mammals including human milk, tears, saliva, mucosal secretions, and the secondary granules of polymorpho-nuclear leucocytes. It was first discovered by
15 Peter Sorenson in 1939 and initially named "red protein" of human milk.

The *lactoferrin* (Lf) was isolated and purified from cow's milk at the first time. Since the first discovery, Lf has been isolated and purified in milk of other mammals such as human being, mouse, goat, rabbit, dog, etc. Human milk has a high content of the *lactoferrin*, for example, in the range of 6 to 8 mg/ml during the colostral phase.
20 However, the *lactoferrin* content in human milk is decreased to about 2 mg/ml in the lactiferous phase. If the human milk is infected with bacteria during the lactiferous phase, the *lactoferrin* content in the infected milk is abruptly raised more than 30 times as high as the normal *lactoferrin* content.

Human *lactoferrin* (hLf) is a glycoprotein whose molecular weight is 78 kDa,
25 composed of a single polypeptide chain containing 691 amino acids. Here, the single polypeptide chain is composed of a 2-fold internal repeating unit with two folded

globular lobes. That is, human *lactoferrin* (hLf) has C and N lobes constituting C and N terminals, respectively. The two lobes have the very similar structure with a high degree of homology (more than about 40%) between the C and N terminals. With the recent advance of X-ray chrystallography, the three-dimensional structure of *lactoferrin* has
5 been determined such that each of the C and N terminals has a site to bind one iron with high affinity and that one *lactoferrin* molecule reversibly binds two ferric ions (Fe^{+3}) (Anderson et al., 1989). Such a *lactoferrin* is present in either iron-free (i.e., apo-type) or iron-saturated state (i.e., holo-type) depending on whether it binds irons, which in turn determines the biological properties of *lactoferrin*. The apo-type *lactoferrin* is present in
10 normal human milk. All kinds of *lactoferrin* are almost stable under the acid condition in relation to *transferrin* and releases irons at a defined pH value.

Lactoferrin, which is one of non-immunoglobulin protective proteins secreted from exocrine glands, directly or indirectly participates in the anti-bacterial mechanism and thereby affects the anti-viral action. *Lactoferrin* possesses anti-bacterial activities
15 against various microorganisms in the state of *in vitro* and *in vivo*. Especially, *lactoferrin* in the iron-free state (apo-type) has anti-microbial activities against gram-negative bacteria such as *E. coli*, *Kleabsiela pnumoni* and *Aerobacter aerogenes*, because it chelates with Fe^{3+} ions necessary for the microorganisms and thus inhibits the growth of the microorganisms. According to an *in vitro* experiment, it has been demonstrated
20 that *lactoferrin* had a potent anti-bacterial activity like antibiotics against 99.99% of bacterium such as *Bacillus*, *E. coli* and *Salmonella* in one hour. *Lactoferrin* also participates in mechanisms involving an iron-binding action to inhibit the growth of microorganisms and abruptly deteriorate the bacteria viability. In the mechanisms, *lactoferrin* not only damages the outer membrane of gram-negative bacteria to release a
25 large amount of lipopolysaccharide constituting the outer membrane and thereby destroy the permeability barrier of the membrane but also increases the sensitivity of the

microorganisms to hydrophobic antibiotics such as lysozyme or rifampicin, thus reducing the resistance of the microorganisms to antibiotics. In spite of the anti-bacterial action against pathogenic organisms, it is reported that *lactoferrin* has no anti-bacterial action against bacterium beneficial to human such as *Lactobacillus* or *Bifidus*. Thus *lactoferrin* contributes to protection of newborn babies against microbial infection. Furthermore, *lactoferrin* is also found in a small amount in blood and secreted from neutrophil. Such a *lactoferrin* is a principal constituent of the secondary granules of the neutrophil and is secreted in a large amount in an inflammatory response. There are some cases that the *lactoferrin* directly has a synergy effect with lysozyme or IgA in living bodies against infected pathogenic microorganisms. As the *lactoferrin* plays an important role in the protective mechanism against infectious hosts, patients who cannot produce *lactoferrin* in the body are seriously deteriorated in resistance to various diseases with an increase in the possibility of infection with bacteria or fungi. Besides, the *lactoferrin* serves as a mediator in cell proliferation or iron transport absorption.

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BACKGROUND ART

Despite that *lactoferrin* has the various functions as previously described, many studies have not been made on the human *lactoferrin* because only a small amount of *lactoferrin* is contained in blood or other bodily fluids and there is a limitation in the available quantity of colostrums sample abundant in *lactoferrin*. As human milk is regarded as a great importance, careful studies on the *lactoferrin* are being made to use a genetic engineering approach to establish the base for industrial application of microorganisms in regard to cloning and expression of human *lactoferrin* (hLf) DNA in microorganisms. However, as described above, there is still a difficulty in using *E. coli* used widely in genetic engineering as an expression strain for human *lactoferrin* (hLf) unless a special recombinant plasmid for *E. coli* is produced.

It is also of great importance to verify the biological activity of the produced recombinant *lactoferrin*, since the normal bacteria or yeasts are mostly improper as a host due to the anti-bacterial activity of *lactoferrin* itself and, if used as a host, inadequate to industrial uses in such a small quantity available. To solve this problem, Ward et al. (1992) successfully produced the recombinant *lactoferrin* using fungi such as *Aspergillus indulans* or *oryzae*. But, there was a limitation in that the *lactoferrin* was expressed in such a small amount of 5 to 25 mg/l in relation to the *lactoferrin* expressed from an insect cell. Furthermore, because the recombinant *lactoferrin* produced from the fungi was analyzed for its biological activity only in terms of affinity with Fe^{58} labeled with radioactive isotopes, there was still remained a question as to whether the recombinant *lactoferrin* actually had an anti-bacterial action on the pathogenic microorganisms.

Meanwhile, studies concerning production of human *lactoferrin* using cell of the higher animals were attempted, but because of the high cost concerning the cultivation process, it is said that the application is difficult.

Thus, researchers in this art have shown some interest in producing human *lactoferrin* using insects cell which can be massively produced at a low cost. For example, it was reported that there were studies done in producing human *lactoferrin* using insects cell (Salmon et al., "Characterization of Human Lactoferrin Produced in the Baculovirus Expression System" in Protein Expr. Purif., vol. 9(2), 203-210, 1997). However, with such method, obtained human *lactoferrin* were very small amount of 15 mg/l. It was estimated that because of the sequence of human *lactoferrin* secretion did not well worked on insect cell. Also, 5% calf serum was used in such method, as a result, purification of human *lactoferrin* was bad.

To overcome the above problems, the present inventor has contrived a novel

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method for mass-production of human *lactoferrin* in a simple way and a verification method for the biological activity of the recombinant human *lactoferrin*.

It is, therefore, an object of the present invention to provide a method for massively producing human *lactoferrin* using an insect cell.

It is another object of the present invention to provide a recombinant insect cell for producing a human *lactoferrin* protein.

It is still another object of the present invention to provide a biological verification method for a recombinant *lactoferrin* produced from an insect cell.

DISCLOSURE OF INVENTION

The present invention relates to a method for producing human *lactoferrin* comprising the steps of transducing a human *lactoferrin* gene into an insect cell by gene recombination, cloning and expressing human *lactoferrin* in the insect cell, and using the insect cell to produce the human *lactoferrin*. The present invention also relates to a

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verification method for the biological activity of the recombinant human *lactoferrin*.

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The present invention is related to a method for producing human *lactoferrin* comprising the steps of transducing a human lactoferrin gene into an insect cell by gene recombination, cloning and expressing human lactoferrin in the insect cell, and using the insect cell to produce the human lactoferrin. The present invention is also related to a
15 verification method for the anti-bacterial activity of the recombinant human *lactoferrin*.

Reference will now be made to Figs. 1 and 2 as to a method for producing human *lactoferrin* using an insect cell according to the present invention.

The novel method for producing human *lactoferrin* by using an insect cell comprises the following steps: (a) combining a transfer vector 1 with a recombinant
20 plasmid pHf-8 2 to prepare a recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK; (b) cotransfecting the recombinant expression vector together with a help vector pBacPAK6 4 into an insect cell Sf9 5 in a culture medium to prepare a recombinant insect cell Sf-Lf 6, and producing a recombinant insect virus from the
25 recombinant insect cell; and (c) producing human *lactoferrin* from the recombinant insect cell Sf-Lf 6.

In the step of producing the recombinant insect cell, the culture medium in which the recombinant insect cell has been cultured is subjected by centrifugal separation to obtain a progeny virus originated from the insect cell.

To prepare the recombinant insect virus, the transfer vector 1 is first combined
5 with the recombinant plasmid 2 to produce the recombinant expression vector pBacLf 3 modified to permit the regulation of a *lactoferrin* gene by a polyhedrin promoter in a vector pBacPAK, and the recombinant expression vector is cotransfected with the help vector pBacPAK6 4 into the insect cell Sf9 5 in a culture medium to produce the recombinant insect cell Sf-Lf 6, from which the recombinant insect virus is produced.

10 The most widely used insect cell is *Spodoptera frugiperda* (Sf9) originated from army worm. The Sf9 cell line is also used as a host cell in the present invention. The Sf9 cell line, if infected with an insect virus such as *Baculovirus*, promotes synthesis of a mucous protein called polyhedrin. This means that the polyhedrin promoter for synthesizing polyhedrin in the *Baculovirus* is highly activated. It is stated in the related
15 reports that the polyhedrin was produced in the insect cell in an amount of 1 to 500 mg/l and that the concentration of the external protein expressed depended on the type of protein or gene (Kaplan et al., 1990; Davidson et al, 1990; and Kaplan et al., 1991). The insect cell was highly analogous to the higher animal cells such as mammalian cells in terms of glycoprotein, phosphorylation, fatty acid acylation, amidation, and proteolytic
20 processing, so that most higher animal cell proteins expressed in the insect cell had biological activities. The human *lactoferrin* as used herein was also verified to have a biological activity.

The expression vector used in the present invention is an insect virus DNA and the most widely used expression vector is multiple polyhedrosis virus AcMNPV called
25 *Autographa californica*. The life cycle and the infection cycle of the *Baculovirus* have been reported in the related document, King L.A and R.D. Possee, The *Baculovirus*

Expression System, A laboratory guide, HAPMAN and HALL. As stated above, the present invention uses an expression vector (pBacPAK, Clontech) originated from *Baculovirus*.

In the present invention, there are used various biochemical and molecular biological methods in order to verify the production of human *lactoferrin* from the insect cell. For example, PCR, Southern blot analysis and Western blot analysis are performed to support a fundamental study for producing a gene recombinant *lactoferrin* in the molecular level.

According to the present invention, the recombinant *lactoferrin* produced from the insect cell is biologically verified in a new way. That is, the recombinant *lactoferrin* is extracted from the insect cell and mixed with pathogenic bacteria such as *Pseudomonas cepacia*, *Pseudomonas putida*, *Pseudomonas fluorescence*, *Salmonella typhimurium* and *E. coli*, after which it is observed how much of the pathogenic bacteria have been destroyed in one hour at maximum.

On the other hand, the recombinant insect cell Sf-Lf used in the present invention is considered as an ideal system for mass production of human *lactoferrin* because it can be cultured in a flask due to its susceptibility of suspension culture and requires neither CO₂ unlike the higher animal cells nor fetal bovine serum (FBS) in cultivation.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Further objects and advantages of the invention can be more fully understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a flow chart showing a process of producing a recombinant human *lactoferrin* using an insect cell according to the present invention.

Fig. 2 is a flow chart showing a process of producing a recombinant expression

vector pBacLf according to the present invention.

Fig. 3 is a photograph showing the electrophoresis pattern of the recombinant expression vector pBacLf after cleavage with a restriction enzyme according to the present invention.

5 Fig. 4 is a photograph of the agarose gel electrophoresis pattern showing that a human *lactoferrin* cDNA has been cloned from the recombinant baculovirus DNA isolated from the recombinant insect cell Sf-Lf according to the present invention.

Fig. 5 is a Southern blot photograph showing that a human *lactoferrin* DNA was cloned from the recombinant virus DNA obtained in Fig. 4.

10 Fig. 6 is a Western blot photograph and SDS-PAGE photograph showing that the recombinant insect cell Sf-Lf expresses and produces the human *lactoferrin* protein.

BEST MODE FOR CARRYING OUT THE INVENTION

EXAMPLE 1: Cultivation of Insect Cell and Production of Recombinant

15 Expression Vector pBacLf

A *Spodoptera frugiperda* ovary cell Sf9 was used as an insect cell and cultured at a low temperature of 28 °C. The insect cell Sf9 was infected with an *autographa californica* nuclear polyhedrosis virus (AcMNPV) (pBacPAK 6) and cultured in a Grace's medium containing 10% FBS, lactalbumine, hydrolysate and antimycotic
20 antibiotics. The insect cell was commercially available from Invitrogen Inc. (PO Box 2312,9704CH Groningen, Netherlands). The virus, AcMNPV (pBacPAK6) was provided by Clontech laboratories Inc. (1020 East Meadow Circle, Palo Alto, C.A. 94303-4230, USA).

To transfer a *lactoferrin* gene into a baculovirus gene, a 2.1 kb full gene including
25 the start codon and the signal sequence of the *lactoferrin* was produced from the existing recombinant plasmid for cloning a transfer vector (pBacPAK8, Clontech Co.) including

the polyhedrin promoter site (5.5 kb), and then inserted into *E. coli* in the same direction of the polyhedrin promoter to prepare a recombinant expression vector. The transfer vector (pBacPAK8) was commercially available from Clontech laboratories Inc. (1020 East Meadow Circle, Palo Alto, C.A. 94303-4230, USA). The recombinant expression
5 vector thus obtained was treated with a restriction enzyme to verify the *lactoferrin* gene, which was named "pBacLf". A process for producing the expression vector pBacLf is illustrated in Fig. 2.

Fig. 3 is a photograph showing the electrophoresis pattern of a selected recombinant expression vector pBacLf after cleavage with a restriction enzyme, wherein
10 lane 1 is a size maker (λ /BstE I); lane 2 is the super coil of the recombinant expression vector pBacLf; lane 3 is the recombinant expression vector pBacLf cleaved with restriction enzymes BamH and Not I to verify the cleaved *lactoferrin* gene at 2.1 kb in terms of the full size of *lactoferrin*; lane 4 is the recombinant expression vector treated with a restriction enzyme, Eco R V to verify the *lactoferrin* gene; lane 5 is the
15 recombinant expression vector treated with a restriction enzyme, Sma I to verify the *lactoferrin* gene; lane 6 is the recombinant expression vector treated with a restriction enzyme, Bgl II to verify the *lactoferrin* gene; lane 7 is the recombinant expression vector treated with a restriction enzyme, Pst I to verify the *lactoferrin* gene; and lane 8 is a size marker. As shown in Fig. 3, a human *interferrin* gene shows at 2.1 kb when the
20 recombinant expression vector was cleaved with restriction enzymes BamH I and Not I into a fragment

EXAMPLE 2: Selection of a Recombinant Insect Cell Sf-Lf and Identification of a Recombinant Virus

Sf9 cells were inoculated in an amount of about 1.0×10^6 cells in a Grace's basic
25 medium containing 10% FBS and were cultured for 4 hours. The insect cell was washed with the Grace's basic medium twice and remained at the ambient temperature for

30minutes. A mixture of a virus DNA (BacPAK 6, Clontech Co.) and a recombinant transfer vector prepared for liposome-mediated transfection, together with lipofectin was dropped on a cell monolayer. The mixture was added to the Grace's medium containing serum and antibiotics and was cultured at 28 °C for 5 days. The supernatant was diluted
5 with the culture solution in ten stages, 3 to 5 times and was inoculated into the insect cell Sf9 cultured in a monolayer on a 60mm-diameter plate. When the virus was adsorbed, the dissolved agarose-containing medium was hardened on the insect cell. After 6 to 7 days, the insect cell with the agarose-containing medium was dyed with neutral red, which dyes dead cells distinguished and forms a plaque. A microscope was used to select
10 a plaque in which the polyhedrin by the infection with the recombinant virus was not formed. The plaque with the agarose was sucked up with a Pasteur pipette and was suspended in a 1ml medium. In order to verify whether the recombinant virus contains the *lactoferrin* gene, another insect cell Sf9 cultured in a new Grace's medium was infected with the recombinant virus and the recombinant virus DNA was isolated, after
15 which the electrophoresis patterns of the isolated DNA were compared by agarose gel electrophoresis. By polymerase chain reaction (PCR) using a primer capable of amplifying *lactoferrin*, it was identified and after treatment with a restriction enzyme, the *lactoferrin* gene (2.1 kb) was identified by Southern blot analysis.

Fig. 4 shows that the *lactoferrin* cDNA (2.1 kb) was cloned from the recombinant
20 virus DNA isolated from the recombinant insect cell Sf-Lf, in which lane 1 shows a size marker (λ /BstE II); lane 2 is a negative control (pBacPAK8); and each of lane 3 and 4 is a recombinant virus DNA. It is seen from lane 3 that there has been amplified the human *lactoferrin* cDNA (2.1 kb) using a primer exclusively amplifying the human *lactoferrin* from the recombinant virus DNA.

25 Fig. 5 verifies the *lactoferrin* DNA (2.1 kb) by Southern blot analysis for the recombinant virus DNA treated with restriction enzymes such as BamH I, Not

I and Acc I, based on the fact verified in Fig. 4, in which lane 1 shows a recombinant virus intact DNA; lane 2 is a recombinant virus DNA (treated with BamH I/Not I); lane 3 is a recombinant virus DNA (treated with Acc I); lane 4 is a recombinant virus DNA (treated with BamH I/Not I/Bgl II); lane 5 is an amplified PCR *lactoferrin* gene produced from the virus DNA; lane 6 is a DIG-labeled size marker; lane 7 is a super coil DNA of the recombinant plasmid (pBacPAK8); and lane 8 is a negative control (pGEMLf) (treated with BamH I/Not I).

The negative control was pBacPAK8 and the positive control was lactoferrin-gene-containing pGEMLf cleaved with a restriction enzyme BamH I/Not I. The probe as used herein was a part of the N-lobe of DIG-labeled *lactoferrin* cDNA. As shown in Fig. 5, the band was observed at the same location (2.1 kb) of the positive control in the recombinant baculovirus DNA. It demonstrates that the selected recombinant virus of the present invention contained human *lactoferrin* DNA.

EXAMPLE 3: Expression of Human *Lactoferrin* from Recombinant Insect Cell (Sf-Lf) and its Verification

To verify expression of protein, the recombinant cell was pulverized with a cell lysis buffer (50 mM Tris-HCl, pH 8.0, 5% 2-mercaptoethanol, 0.4% w/v SDS, 10 mM EDTA) and applied to coomassie-blue polyacrylamide gel running, after which a Western blot analysis was performed using anti-*lactoferrin* (anti-Lf).

As shown in Fig. 6, the recombinant viral stock was inoculated into the insect cell Sf9 and the cells were pulverized with the insect host cell Sf9 at the fourth day. The supernatant was collected for a Western blot analysis with anti-*lactoferrin* (anti-Lf) Ab and SDS-PAGE and, as a result of which the band was color-developed at the same location (80 kDa) of colostrums used as a positive control and purified *lactoferrin* protein. This demonstrates that the human *lactoferrin* protein was produced in the recombinant

insect cell (Sf-Lf). Lane 1 shows a protein size marker, lane 2 is a colostral soup, lane 3 is the insect cell Sf9, lanes 4 and 5 are the recombinant insect cells. A densitometry revealed that the expressed *lactoferrin* was in an amount of more than 800 mg/l. The amount expressed is much higher than the amount of *lactoferrin* expressed from 5 *Aspergillus Nidulans* or *Aspergillus Oryzae* and shows economically high productivity.

EXAMPLE 4: Verification of Anti-bacterial Activity of Recombinant *Lactoferrin*

To measure the anti-bacterial activity of the recombinant insect cell (Sf-Lf) against pathogenic microorganisms, the insect cell (Sf-Lf) was pulverized by a freeze and thaw method and the supernatant was mixed with the pathogenic microorganisms in the 10 *lactoferrin* concentration of about 250 µg/ml. The mixture was then smeared to a plate count agar plate at intervals of 0, 15, 30, 45 and 60 minutes.

It can be seen from the cell count of Table 1 that the *lactoferrin*-containing the supernatant alone destroys the pathogenic microorganisms within one hour. For the negative control, insect cell (Sf) was pulverized and subjected to microassay on 15 pathogenic microorganisms in the same way as for the recombinant insect cell (Sf-Lf), in which case there was no decrease in the number of cells.

The above results revealed that the *lactoferrin* protein produced from the recombinant insect cell (Sf-Lf) possessed an anti-bacterial activity.

20 INDUSTRIAL APPLICABILITY

The recombinant insect cell (Sf-Lf) produced by the method for producing human *lactoferrin* using an insect cell according to the present invention is advantageous in that it can be cultured in a flask due to its susceptibility of suspension culture, requiring neither carbon dioxide (CO₂) unlike the higher animal cells, nor fetal bovine serum 25 (FBS) in the culture, as a result of which using the recombinant insect cell allows mass production of human *lactoferrin* at a low cost in a simple way.

Table 1

Strain	Cell Count (cfu/ml)									
	* <i>E. coli</i> 300		* <i>S. typhimurium</i> 114		@ <i>P. putida</i>		@ <i>P. fluorescens</i>		@ <i>P. cepasia</i> 9613	
	-	+	-	+	-	+	-	+	-	+
0 min	>10 ⁷	>10 ⁷	>10 ⁷	>10 ⁷	>10 ⁷	6.7×10 ⁶	>10 ⁷	1.8×10 ⁶	5×10 ³	1.4×10 ³
15 min	>10 ⁷	>10 ⁵	>10 ⁷	5.0×10 ⁴	>10 ⁷	3.0×10 ⁴	>10 ⁷	1.4×10 ⁴	5×10 ³	9.8×10 ²
30 min	>10 ⁷	1.3×10 ⁴	>10 ⁷	1.2×10 ⁴	>10 ⁷	6.9×10 ³	>10 ⁷	4.0×10 ³	5×10 ³	2.1×10 ²
45 min	>10 ⁷	6.2×10 ³	>10 ⁷	2.0×10 ³	>10 ⁷	3.0×10 ³	>10 ⁷	2.9×10 ³	5×10 ³	10
60 min	>10 ⁷	10 ²	>10 ⁷	1.2×10 ²	>10 ⁷	10 ²	>10 ⁷	5×10 ²	5×10 ³	0

Note: * indicates an animal pathogenic bacterium; @ indicates a food contaminant bacterium; + indicates a culture with *lactoferrin*; and – indicates a culture without *lactoferrin*

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